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①⑮ **Method of assaying physiologically active substances.**

①⑮ A method of assaying a physiologically active substance, which comprises activating blood coagulation factor XII in the plasma in the presence of said physiologically active substance being tested to convert prekallikrein in the plasma into kallikrein, and measuring the activity of kallikrein thus formed.

EP 0 259 857 A2

METHOD OF ASSAYING PHYSIOLOGICALLY ACTIVE SUBSTANCES

BACKGROUND OF THE INVENTION

1. Field of the Invention

This invention relates to a method of assaying physiologically active substances. More particularly, it relates to a method of assaying physiologically active substances involved in the process of plasma kallikrein formation.

2. Description of the Prior Art

Kallikrein is a group of protease widely distributed in the plasma and tissues of animals, and is known to participate in an enzyme reaction system called kallikrein-kinin system

This kallikrein-kinin system is closely related to many other enzyme reaction systems, such as the renin-angiotensin system, the blood coagulation system, the fibrinolysis system, the complement system, and the arachidonate cascade producing prostaglandin, leukotriene and thromboxane, as well as to the behavior of catecholamine, thus playing an important role for function control in living bodies. To be more specific, it is deeply concerned, through other enzyme reaction systems, with blood pressure control, control actions relating to blood coagulation fibrinolysis-complement system, and peripheral blood circulation improving and other control actions exerted by various physiologically active substances produced from the arachidonate cascade.

It is known that plasma kinin, which is a product of the kallikrein-kinin system, shows various physiological actions, such as hypotensive action as a result of vasodilation, enhancement of vascular permeability, contraction and relaxation of smooth muscles, development of pains, leucocytes chemotaxis, and liberation of catecholamine from the adrenal cortex. It is also known that plasma kinin acts as a mediator for acute inflammations, including allergic reactions. Thus the presence of plasma kinin in living bodies is of great significance.

Hence, establishing a simple method for correctly evaluating the action of substances involved in the formation of kallikrein (substances that retard or accelerate the formation of kallikrein) would be of great use in learning the aforementioned actions for function control in living bodies and also in developing new drugs having such actions.

The kallikrein-kinin system involves a series of enzyme reactions as described below.

Blood coagulation factor XII (Hageman factor; hereinafter abbreviated as F-XII) is the substance which plays the major role in this enzyme reaction system. F-XII in the plasma is activated upon contact with a negatively charged substance (such as glass, kaolin and elaidic acid) or with a substance existing in living bodies (such as collagen, homocystine, platelet membrane and sulfated glycolipid), or as a result of nociceptive stimulation upon tissues. The resulting activated F-XII (F-XIIa) then acts upon prekallikrein present in the plasma to convert it into kallikrein, which in turn acts upon high-molecular-weight in the plasma, liberating bradykinin which is a nonapeptide.

Eventually, the kinin thus formed causes inflammations and pains, and exerts various influences on living bodies through action upon the arachidonate cascade.

In-vitro simulation of the aforementioned series of enzyme reactions related to the kallikrein-kinin system has led us to find that the reaction system adopted in this invention provides a simple, highly reliable, and more effective method for assaying physiologically active substances. This invention was accomplished based on these findings.

SUMMARY OF THE INVENTION

An object of this invention is to provide a method for assaying physiologically active substances involved in the formation of kallikrein.

Another object of this invention is to provide a method for determining whether a substance (a drug) is involved in the formation of kallikrein or not.

A further object of this invention is to provide a method for measuring the extent to which a physiologically active substance is involved in the formation of kallikrein.

A still further object of this invention is to provide a reaction system best suited to the method mentioned above.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graph showing the inhibitory actions of various analgesics against the formation of kallikrein measured by the method of this invention, and Figure 2 is a graph showing their inhibitory actions against the liberation of bradykinin.

DETAILED DESCRIPTION OF THE INVENTION

This invention relates to a method of assaying a physiologically active substance, which comprises activating F-XII in the plasma in the presence of said physiologically active substance (a drug being tested) to convert prekallikrein contained in the plasma into kallikrein by the action of F-XIIa thus formed, and measuring the amount of the resulting kallikrein.

More specifically, this invention relates to a method of assaying a physiologically active substance, which comprises allowing a reaction to proceed by mixing (1) animal plasma, (2) an activator for blood coagulation factor XII, (3) an electrolyte and (4) a drug being tested; adding an inhibitor having practically no action upon the kallikrein activity and capable of specifically inhibiting the activity of the activated blood coagulation factor XII; and measuring the amount of kallikrein thus formed.

In a preferred embodiment of this invention, a reaction is allowed to proceed by mixing (1) animal plasma, (2) an activator for blood coagulation factor XII, (3) an electrolyte and (4) a drug being tested, and an inhibitor having practically no action upon the kallikrein activity and capable of specifically inhibiting the activity of the activated blood coagulation factor XII is then added. To the resulting mixture, was added a solution of a substrate for kallikrein in a buffer solution, and the amount of decomposition product formed by the action of kallikrein was measured, thereby determining the efficacy of drug being tested.

The reaction system adopted in the method of this invention involves two steps as described above. The primary reaction is a step in which F-XII in the plasma is converted into F-XIIa by addition of an activator, such as kaolin, which in turn acts upon prekallikrein to form kallikrein. The secondary reaction that follows is a step in which the amount of kallikrein formed is measured; for example, the activity (or quantity) of kallikrein may be determined by the use of a substrate specific to kallikrein.

Thus, the method of this invention is characterized in that a physiologically active substance involved in the formation of kallikrein is allowed to be present in the primary reaction system mentioned above, and its quantity is determined in the secondary reaction system.

In the method of this invention, plasma of any type of animal may be used so long as it contains the blood coagulation and kallikrein-kinin systems. This includes plasma of humans, monkeys, cattle, sheeps, pigs, horses, goats, dogs, cats, rabbits, guinea pigs, rats and mice. Of these, the plasma of humans and rats are the most preferred.

Plasma used in the method of this invention may be prepared by any known methods. For example, a blood sample taken in the presence of sodium citrate is centrifuged and the resulting supernatant may be used as citrate-containing plasma. Freeze-dried plasma prepared by usual method may also be used for the purpose of this invention.

The animal plasma thus prepared may be appropriately diluted, as required, before use. Its concentration should be within the range over which a linear relationship holds between its dilution and the kallikrein activity.

A variety of substances may be used as the activator for F-XII in the method of this invention. These include kaolin, collagen, dextran sulfate, elaidic acid and Celite. To ensure satisfactory activation of F-XII, each of these activators should be used at its optimum concentration. For example, when an aqueous suspension of kaolin is used for human plasma, its final concentration should preferably be in the range of 1 to 3 mg/ml, most preferably, in the range of 1.15 to 2 mg/ml.

The electrolyte is added to make the action of the F-XII activator perfect. Preferable examples are those containing monovalent cation (e.g., sodium), such as sodium chloride, and sodium acetate.

With human plasma, for example, the final concentration of sodium chloride in the primary reaction should preferable be in the range of 50 to 200mM, most preferably, in the range of 75 to 150mM.

If the primary reaction is allowed to proceed at an elevated temperature, it progresses too rapidly and at the same time endogenous inhibitors are put to work, thus significantly affecting the measurement of kallikrein activity. It is therefore preferable to carry out the primary reaction at a low temperature (for example, at 0 to 4°C) so that it will proceed slowly and endogenous inhibitors will not work.

- 5 The primary reaction should preferably be carried out at the optimum pH for kallikrein which is the final product of this reaction step; when human or rat plasma is used, the suitable pH is in the range of 7.0 to 9.0, most preferably, in the range of 7.5 to 8.5.

The suitable reaction time may vary with the amounts of plasma and F-XII activator added, the concentration of the drug being tested and the pH of the reaction system, but it should be set within the
10 range over which a linear relationship holds between the reaction time and the amount of kallikrein formed (or kallikrein activity). This is because the method of this invention assays the activity involved in the formation of plasma kallikrein of a physiologically active substance as kallikrein activity and hence activity measurement must be made at a moment before the amount of kallikrein becomes saturated and when the aforementioned linear relationship still holds. In actual practice, however, it is preferable to set the reaction
15 time within the range of 15 to 30 minutes.

The primary reaction may be terminated by addition of an inhibitor which specifically inhibits the activity of activated F-XII to prevent formation of extra kallikrein and shows practically no effect upon the activity of kallikrein to be measured in the secondary reaction.

- As examples of such inhibitors, there may be mentioned LBTI (Lima Bean Trypsin Inhibitor) and CHFI
20 (Corn Hageman Fragment Inhibitor). These inhibitors should be added at the end of the primary reaction in such an amount as to completely inhibit the activity of F-XIIa left in the primary reaction mixture and as to show practically no effect upon measurement of Kallikrein in the secondary reaction. When human plasma is used, for example, LBTI should preferably be added at a final concentration of 4 to 15 mg/ml.

As described above, the secondary reaction is a step for measuring the amount of kallikrein formed in
25 the primary reaction, preferably by the use of a substrate specific to kallikrein.

- A variety of substances may be used as the substrate specific to kallikrein. These include high-molecular-weight kininogen present in the plasma, and synthetic substrates such as N-benzoyl-N-arginine ethyl ester (Benzoyl-Arg-OEt), N-tosyl-L-arginine methyl ester (Tos-Arg-OMe), D-prolylphenylalanylarginyl-p-nitroanilide (D-Pro-Phe-Arg-pNA). benzoyl prolylphenylalanylarginyl-p-nitroanilide (Benzoyl-Pro-Phe-Arg-pNA), prolylphenylalanylarginyl-naphthylamide (Pro-Phe-Arg-NA) and
30 benzoyloxycarbonyl-phenylalanylarginyl-4-methylcoumarinamide (Z-Phe-Arg-MCA).

When high-molecular-weight kininogen is used as the substrate, bradykinin thus formed can be determined by commonly employed techniques - through bioassay using smooth muscles like the guinea-pig ileum or rat myometrium (Magnus method), or through radioimmunoassay (RIA).

- When the activity of kallikrein is measured by utilizing its esterase action, Benzoyl-Arg-OEt or Tos-Arg-
35 OMe may be used as the substrate. The quantity of kallikrein may be determined by various methods. These include measuring the change in absorbance due to hydrolysis; spectrophotometric determination after conversion into a colored derivative [e.g., the hydroxamate method and the method using chromotropic acid or MBTH (3-methyl-3-benzothiazolonehydrazine)]; fluorometric determination after
40 conversion into a fluorescent derivative; the method using alcohol dehydrogenase; and radiochemical determination by the use of a radioactive synthetic substrate.

In addition, D-Pro-Phe-Arg-pNA, Benzoyl-Phe-Phe-pNA and Z-Phe-Arg-MCA may also be used as a colored or fluorescent, synthetic peptide substrate. In this case, the activity of kallikrein can be determined by spectrophotometric measurement of the colored or fluorescent substance formed by its hydrolytic action,
45 for example, pNA (p-nitroaniline) and AMC (7-amino-4-methylcoumarin).

In the secondary reaction, the pH should preferably be maintained in the range of 7.0 to 9.0 (in the vicinity of the optimum pH for kallikrein), and the reaction temperature be maintained in the range of 20 to 40°C (in the vicinity of the optimum temperature for kallikrein; room temperature). The suitable reaction time may vary with the temperature, pH and substrate concentration, but it should be set within about 30
50 minutes to ensure high operation efficiency.

EXAMPLE 1

- 55 An example in which human plasma was used as animal plasma is detailed below.

1. Preparation of citrate-containing human plasma

A blood sample, taken from a healthy adult in the presence of sodium citrate according to the commonly used procedure (human blood:3.8% sodium citrate = 9:1), was centrifuged, and the supernatant was collected as citrate-containing human plasma (hereinafter referred to simply as human plasma).

(2) Primary reaction

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Human plasma	0.1 ml
Aqueous suspension of kaolin	0.5 ml
Aqueous solution of sodium chloride	} 0.4 ml
Aqueous solution of drug being tested	
Distilled water	

In the above formulation, the human plasma had been diluted with physiological saline to such a concentration that kallikrein activity of 1.6 to 2.2 mU will be shown in the secondary reaction. For kallikrein activity, its amount that produces 1 μ mole/min/ml of p-nitroaniline in the secondary reaction was taken as 1 U (1,000 mU).

The concentration of kaolin suspension was 2.5 mg/ml [in 50mM Tris-HCl buffer (pH 8.0)], and that of sodium chloride in the mixed solution (0.4 ml) was 0.25M.

The above mixture was allowed to stand in an ice/water bath for 20 minutes to effect the primary reaction, which was terminated by addition of 0.5 ml LBTI solution [45 mg/ml in 50mM Tris-HCl buffer (pH 8.0)] to make the primary reaction mixture. It may also be prepared by taking 0.2 ml of the reaction mixture before termination and adding it to 0.1 ml of LBTI solution.

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(3) Secondary reaction

Primary reaction mixture	0.1 ml
Synthetic substrate	0.1 ml
Buffer solution	0.2 ml

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In the above formulation, the synthetic substrate was 4mM aqueous solution of D-Pro-Phe-Arg-pNA, and the buffer solution was 100mM Tris-HCl buffer (pH 8.0).

The above mixture was allowed to stand at 30°C for 20 minutes to effect the secondary reaction, 0.8 ml of 1% citric acid was added, suspended solids, if any, were removed by centrifugation, and the amount of p-nitroaniline was determined by measuring the absorbance at 405nm.

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(4) Optimum pH for the primary reaction

The primary reaction was carried out using 50mM Tris-HCl buffer solutions of different pH levels. The activity of kallikrein thus formed was measured in the succeeding secondary reaction, which showed that the optimum pH for the primary reaction lies within the range of 7.0 to 9.0, particularly, within the range of 7.5 to 8.5.

(5) Optimum sodium chloride concentration in the primary reaction

The primary reaction was carried out using sodium chloride solutions of different concentrations. The activity of kallikrein thus formed was measured in the succeeding secondary reaction, which showed that the optimum final concentration of sodium chloride in the primary reaction lies within the range of 50 to 200mM, particularly, within the range of 75 to 150mM.

Note that the amount of sodium chloride contained in plasma is not included in the above concentrations.

(6) Optimum kaolin concentration in the primary reaction

The primary reaction was carried out in 50mM Tris-HCl buffer solution (pH 8.0) using kaolin suspension of different concentrations. The activity of kallikrein thus formed was measured in the succeeding secondary reaction, which showed that the optimum final concentration of kaolin lies within the range of 1 to 3 mg/ml, particularly, within the range of 1.25 to 2 mg/ml.

(7) Optimum reaction time for the primary reaction

The primary reaction was carried out over different periods of time, and the activity of kallikrein thus formed was measured in the succeeding secondary reaction. It was found that a linear relationship between the reaction time and kallikrein activity holds within the reaction time range of 0 to 20 minutes, indicating that the primary reaction time should not exceed 20 minutes.

(8) Amount of LBTI used in the primary reaction

The primary reaction was carried out at 0°C for 20 minutes, LBTI solutions of different concentrations were added, and 0.1 ml of each reaction mixture was subjected to the secondary reaction to measure the kallikrein activity.

It was demonstrated that LBTI, if added at a final concentration of 4 mg/ml or more, completely inhibits the action of F-XIIa. No significant drop in kallikrein activity was observed with LBTI of up to 15 mg/ml concentration.

(9) Effect of human plasma concentration in the primary reaction

The primary reaction was carried using human plasma of different dilutions, and the activity of kallikrein thus formed was measured in the succeeding secondary reaction. A linear relationship was observed between the dilution and kallikrein activity within the dilution range of 1/5 to 1/10.

(10) Substrate concentration in the secondary reaction

The secondary reaction was carried out at 30°C for 20 minutes using, as synthetic substrate, D-Pro-Phe-Arg-pNA of different concentrations. The K_m value for the secondary reaction was found to be 0.34mM.

(11) Amount of enzyme used in the secondary reaction

The secondary reaction was carried out using different amounts of enzyme (namely, different amounts of the primary reaction mixture) to study the relationship between the amount of enzyme and kallikrein activity. A linear relationship was observed with the amount of the primary reaction mixture up to 0.1 ml.

(12) Reaction time for the secondary reaction

The secondary reaction was carried out over different periods of time to study the relationship between the reaction time and kallikrein activity. A linear relationship was observed within the reaction time range of 0 to 20 minutes, indicating that the secondary reaction time should not exceed 20 minutes.

(13) Optimum pH for the secondary reaction

The secondary reaction was carried out in 100mM Tris-HCl buffer solutions of different pH levels to study the relationship between the pH and kallikrein activity. It was demonstrated that the optimum pH for the secondary reaction lies within the range of 7.0 to 9.0, particularly within the range of 7.5 to 8.5

Example 2

Described below is an example which employed human plasma as animal plasma, CHFI as specific inhibitor against F-XIIa, and Z-Phe-Arg-MCA as specific substrate for kallikrein.

(1) Primary reaction

The same formulation as in EXAMPLE 1 was used in this case too.

The reactant mixture was allowed to stand for 20 minutes in an ice/water bath to effect the primary reaction, which was terminated by addition of 0.5 ml CHFI solution [3 mg/ml in 50mM Tris-HCl buffer (pH 8.0, 0°C)] to make the primary reaction mixture. It may also be prepared by taking 0.2 ml of the reaction mixture before termination and adding it to 0.1 ml CHFI solution.

(2) Secondary reaction

Primary reaction mixture	0.1 ml
Synthetic substrate	0.12 ml
Buffer solution	0.18 ml

In the above formulation, the synthetic substrate was 10mM solution of Z-Phe-Arg-MCA (a fluorescent substrate) in dimethylsulfoxide, and the buffer solution was 100mM Tris-HCl buffer (pH 8.0).

The above mixture was allowed to stand at 30C for 20 minutes to effect the secondary reaction, 2 ml of 0.1M acetic acid was added, suspended solids, if any, were removed by centrifugation, and the amount of 7-amino-4-methylcoumarin was fluorometrically determined (E_x : 380 nm; E_m : 460 nm).

(3) Conditions for the primary and secondary reactions were studied in the same way as in EXAMPLE 1.

In the primary reaction, the action of F-XIIa could be inhibited by CHFI solutions of 2 mg/ml and higher concentrations.

5 The primary reaction in this case differs from that in EXAMPLE 1 only in that a CHFI solution was used to terminate the reaction, all the other conditions being the same as those in EXAMPLE 1.

In the secondary reaction, the optimum pH was in the range of 7.0 to 9.0, particularly in the range of 7.5 to 8.5, and the K_m value for the synthetic fluorescent substrate, Z-Phe-Arg-MCA, was about 1mM.

10 A linear relationship between the secondary reaction time and kallikrein activity was observed within the reaction time range of 0 to 30 minutes, indicating that the secondary reaction time should not exceed 30 minutes.

EXAMPLE 3

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Detailed below is an example in which freeze-dried human plasma was used as animal plasma.

(1) Primary reaction

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	Freeze-dried human plasma	0.1 ml
25	Aqueous suspension of kaolin	0.5 ml
	Aqueous solution of sodium chloride	} 0.4 ml
30	Aqueous solution of drug being tested	
	Distilled water	

35 The freeze-dried human plasma was dissolved in distilled water and then diluted with physiological saline to such a concentration that kallikrein activity of 1.6 to 2.2 mU will be exhibited in the secondary reaction.

The kaolin suspension, sodium chloride solution, reaction temperature and time, and termination operation were the same as in EXAMPLE 1.

40

(2)

The secondary reaction was carried out in the same manner as in EXAMPLE 1.

45

(3)

50 Studies on the conditions for the primary and secondary reactions made in the same manner as in EXAMPLE 1 showed that freeze-dried human plasma can be used under same conditions as fresh human plasma.

EXAMPLE 4

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Described below is an example in which rat plasma was used as animal plasma.



(1) Preparation of citrate-containing rat plasma

A blood sample, taken from the abdominal aorta of an etherized rat in the presence of sodium citrate (blood:3.8% sodium citrate = 9:1), was centrifuged and the supernatant was collected as citrate-containing rat plasma.

(2) Primary reaction

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Rat plasma	0.1 ml
Aqueous suspension of kaolin	0.5 ml
Aqueous solution of sodium chloride	0.4 ml
Aqueous solution of drug being tested	
Distilled water	

In the above formulation, the rat plasma had been diluted with physiological saline by a factor of 3 to 5, and the kaolin suspension and the sodium chloride solution were the same as used in EXAMPLE 1. The above mixture was allowed to stand at 0°C for 15 minutes to effect the primary reaction, which was terminated by addition of 0.5 ml LBTI solution [45 mg/ml in 50mM Tris-HCl buffer (pH 8.0)].

(3) Secondary reaction

The secondary reaction was carried out in much the same manner as in EXAMPLE 1, except that the reaction was continued for 30 minutes.

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(4) Conditions for the primary and secondary reactions were studied in the same way as in EXAMPLE 1

It was found that, in the primary reaction, the optimum pH lies in the range of 7.0 to 9.0, particularly, in the range of 7.5 to 8.5, the optimum concentration of sodium chloride solution is in the range of 0 to 150mM, particularly, in the range of 25 to 125mM, and the optimum concentration of kaolin suspension is in the range of 1 to 3.5 mg/ml, particularly in the range of 1.5 to 3 mg/ml.

The primary reaction time should preferably be within 20 minutes, because a linear relationship between the reaction time and kallikrein activity was observed within the reaction time range of 0 to 20 minutes.

For the secondary reaction, it was demonstrated that the optimum pH lies in the range of 7 to 9 and the K_m value for D-Pro-Phe-Arg-pNA is 0.29mM.

A linear relationship between the reaction time and kallikrein activity was observed within the reaction time range of 0 to 60 minutes. Hence, the secondary reaction should not exceed 60 minutes, and should preferably be about 30 minutes in actual practice.

EXAMPLE 5

Described below is an example in which dextran sulfate (M.W.: approximately 500,000) was used as the F-XII activator. The other conditions were the same as in EXAMPLE 1, except that fresh human plasma and freeze-dried human plasma were employed as animal plasma.

(1) The optimum final concentration of dextran sulfate was in the range of 1 to 5 μ g/ml, particularly in the range of 1.5 to 3 μ g/ml.

FIG. 1

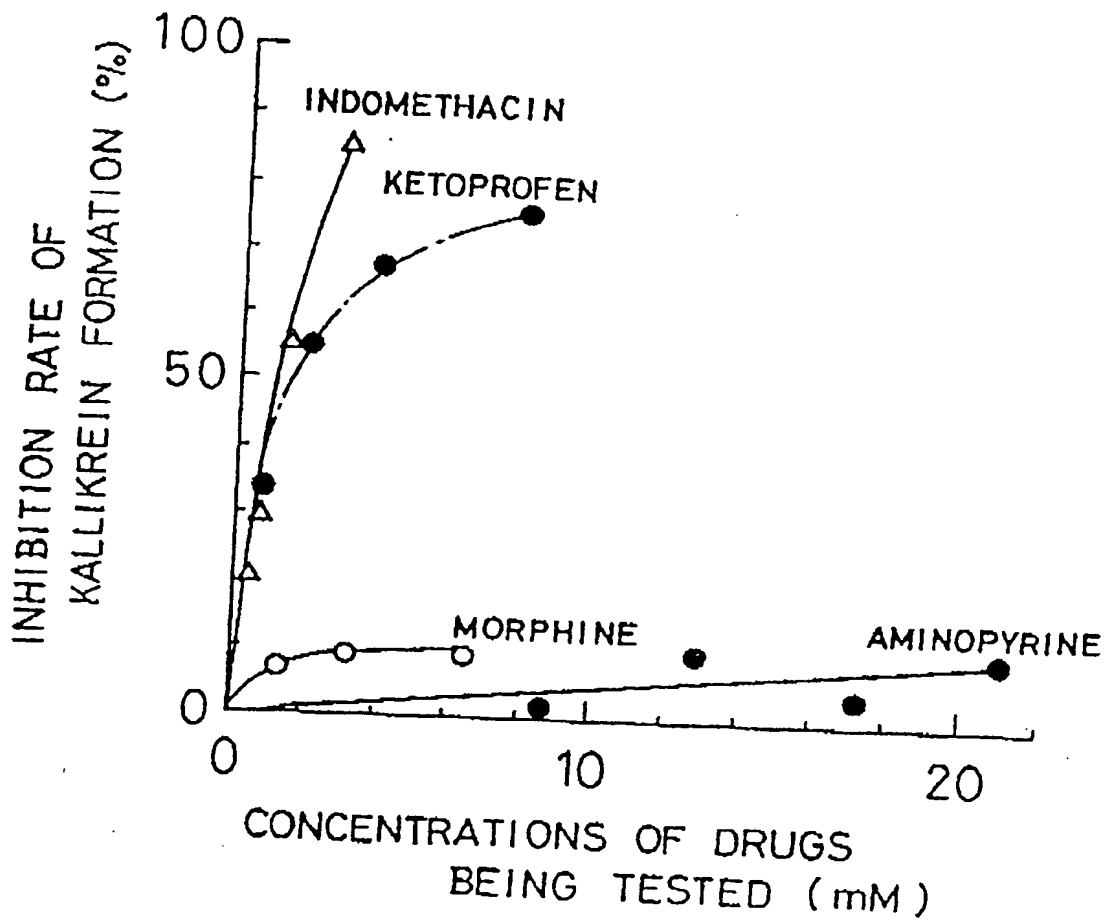
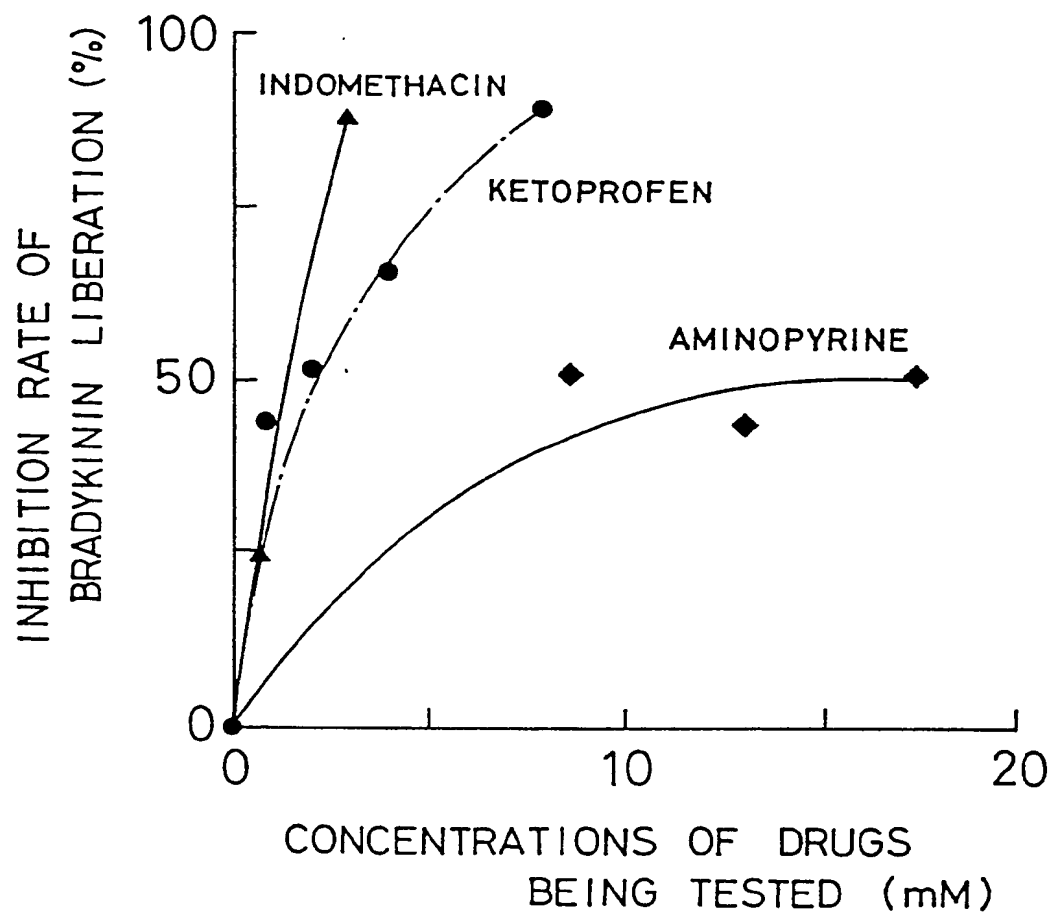


FIG. 2



(19)



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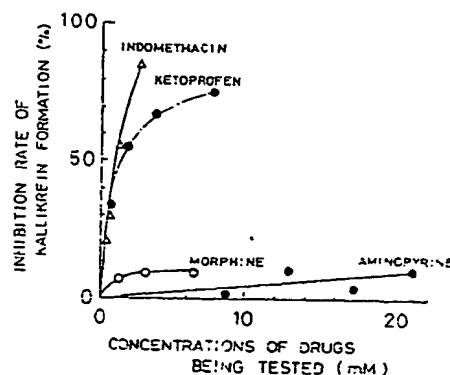
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(54) Method of assaying physiologically active substances.

(57) A method of assaying a physiologically active substance, which comprises activating blood coagulation factor XII in the plasma in the presence of said physiologically active substance being tested to convert prekallikrein in the plasma into kallikrein, and measuring the activity of kallikrein thus formed.

FIG. 1



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A	IDEM * Page 559; figure 3 *	2-8	
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Place of search BERLIN		Date of completion of the search 17-11-1989	Examiner DE KOK A.J.
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X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document		T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons ----- & : member of the same patent family, corresponding document	

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The present search report has been drawn up for all claims			
Place of search BERLIN		Date of completion of the search 17-11-1989	Examiner DE KOK A.J.
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